Assembly and disassembly of the nucleolus during the cell cycle

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The nucleolus is a large nuclear domain in which transcription, maturation and assembly of ribosomes take place. In higher eukaryotes, nucleolar organization in three sub-domains reflects the compartmentation of the machineries related to active or inactive transcription of the ribosomal DNA, ribosomal RNA processing and assembly with ribosomal proteins of the two (40S and 60S) ribosomal subunits. The assembly of the nucleoli during telophase/early G_1 depends on pre-existing machineries inactivated during prophase (the transcription machinery and RNP processing complexes) and on partially processed 45S rRNAs inherited throughout mitosis. In telophase, the 45S rRNAs nucleate the prenucleolar bodies and order the dynamics of nucleolar assembly. The assembly/disassembly processes of the nucleolus depend on the equilibrium between phosphorylation/dephosphorylation of the transcription machinery and on the RNP processing complexes under the control of the CDK1-cyclin B kinase and PP1 phosphatases. The dynamics of assembly/disassembly of the nucleolus is time and space regulated.

Introduction

Eukaryotes have evolved to an « open » form of mitosis in which chromosome segregation is coupled to breakdown and subsequent reassembly of nuclear organization. For more than one century, the nucleolus has served as a model to describe the processes of nuclear disorganization at the beginning of mitosis, transmission of building blocks during mitosis, and reassembly of functional domains at the beginning of interphase.[1] Presently, the control of the different nucleolar assembly/disassembly steps in metazoans and the complexity of these processes are progressively being deciphered.[1,2]

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The nucleolus is a large nuclear domain assembled around the ribosomal genes (rDNAs) when in higher eukaryotes the 47S ribosomal RNAs (rRNAs) are transcribed, processed into 18S, 5.8S and 28S rRNAs, and assembled with the 5S rRNAs and appropriate proteins to yield the small (18S RNAs) and large (5.8S, 28S and 5S RNAs) ribosome subunits.[3] The nucleolus is the ribosome factory of the cell, and proposed as "an organelle formed by the act of building a ribosome."[4] In each eukaryote nucleus, there is at least one nucleolus whose size and organization is directly related to ribosome production.[5-7] In addition to ribosome production, the nucleolus is also a plurifunctional domain involved in stress response, in the biogenesis of ribonucleoprotein particles independently of ribosome subunits, as well as in several diseases including cancer, ribosomopathies or viral infections.[8-11] In particular, the nucleolus has been implicated in assembly of the signal recognition particle (SRP),[12,13] in modification of U2 and U6 spliceosomal small RNA[14,15] and in assembly of specific mRNPs.[16]

During interphase, the different steps of ribosome biogenesis are responsible for the basic structure and general organization of the nucleolus. Three major building blocks, the fibrillar centers (FCs), the dense fibrillar component (DFC) and the granular component (GC) can be identified. These structures reflect the compartmentation of the machineries related to rDNA transcription, rRNA processing and assembly of the two (40S and 60S) ribosomal subunits.[17] Briefly, the initiation of rDNA transcription occurs at the border FC-DFC, early processing of the rRNAs in the DFC and late processing in the GC. Consequently, particular steps of ribosome production can be identified by specific markers of a particular step and localized by light microscopy in the whole cell volume with the advantage of easy access to 3-D information and the possibility of analyzing the dynamics of these molecules in living cells.

During the cell cycle in higher eukaryotes, ribosome production starts at the end of mitosis, increases during G_1, is maximal in G_2[18] and stops during prophase.[19] At the end of mitosis, the machineries necessary to assemble the nucleoli are inherited by
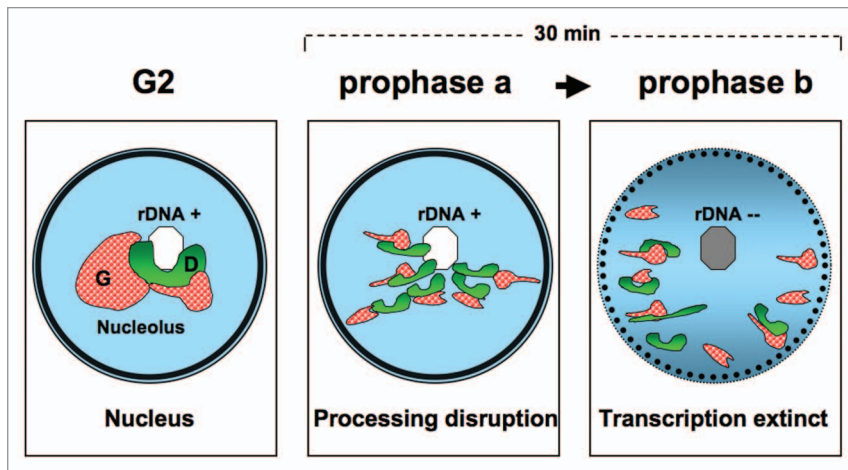
Figure 1. Schematic illustration of nucleolar disassembly at the beginning of mitosis. In the G$_2$ phase of the cell cycle, the nucleolus is composed of 3 structures corresponding to different steps of ribosome biogenesis: (1) the fibrillar center or FC (white octagon) were the ribosomal genes (rDNAs) are localized, (2) the dense fibrillar component or DFC (D in green) corresponding to transcription of the 47S rRNAs and the early steps of the rRNA processing generating the small ribosome subunits and (3) the granular component or GC (G in red) corresponding to late steps of rRNA processing generating the large ribosome subunits. The nuclear envelope (double black rings) is at the periphery of the nucleus surrounding the chromatin (blue). At the beginning of prophase (prophase a), rDNA transcription is still active (white octagon) and the nucleolar processing proteins from the D and G components leave the nucleolus (processing disruption). At the end of prophase (prophase b), rDNA transcription is extinct (grey octagon), the nucleolar processing proteins from the D and G components are dispersed around condensing chromosomes (not shown) and the nuclear envelope is disrupted. In Hela cells the total duration of the prophase is of ≈30 min.

the two daughter cells. In this review, recent progress concerning the timing and the processes controlling assembly/disassembly of the nucleolus during mitosis will be analyzed in mammalian cells and some of the outlooks and perspectives will be proposed.

Disassembly of the Nucleolus at the Beginning of Mitosis

Nucleolar disassembly is a sequential process, starting at the beginning of mitosis with the ordered release of the processing nucleolar complexes followed by the repression of RNA polymerase I (pol I) transcription (**Fig. 1**). It was estimated that pol I transcription decreases by about 30% during early prophase and stops in late prophase.[19,20] At the end of prophase, when nuclear envelope breakdown is achieved[21] and chromosomes are condensed, the nucleolus is no longer visible[22] but the building blocks of the future nucleoli are stored and maintained in different locations during mitosis.

Release of the nucleolar processing complexes. In living cells, dissociation of the NPM/B23 proteins from the nucleolus occurs at the end of prophase before nuclear envelope breakdown.[23] In PtK1 cells at early prophase, the shape of the nucleolus is modified by the release of the nucleolar proteins localized in the GC and DFC. These proteins are distributed in a 3-D network while the chromatin condenses into chromosomes forming a perichromosomal compartment.[24] This perichromosomal compartment is observed in many vertebrates and plant cells supporting the

hypothesis that it is a general behavior of open mitosis.[25] The most representative complexes identified in the perichromosomal compartment correspond to the DFC such as fibrillarin in association with U3 snoRNAs, nucleolin and Nopp140, as well as GC proteins such as NPM/B23, Bop1, Nop52, PM-Scl 100 and Ki67.[1,26-29] Ribosomal proteins (r-proteins), several snoRNAs and different parts of pre-rRNAs including the 45S rRNAs are detected in the perichromosomal compartment.[1,30,31] The colocalization of these different factors in the perichromosomal compartment suggests that early and late rRNA processing complexes are at least partly maintained during mitosis. However the interaction between proteins of the same complexes was not detected by FRET (Fluorescence Resonance Energy Transfer) during anaphase even if the 3-D colocalization of proteins (fibrillarin, Bop1, Nop52 and NPM/B23) was observed around the chromosomes.[26] The interactions between these proteins were detected during nucleolar assembly suggesting a cell cycle regulation of the interactions of the rRNA processing complex (see Nucleolus Assembly below for details). In metaphase-arrested cells, the chromosomes can be isolated with the nucleolar processing complexes still attached.[24] In living cells, the nucleolar proteins tagged with GFP (green fluorescence protein) migrate with the chromosomes during anaphase.[32] These coordinated movements indicate a certain degree of association during this process. How the association between the perichromosomal compartment and the chromosomes is maintained, is presently unknown.

Arrest of rDNA transcription. The production of 47S rRNAs is stopped at the end of prophase while the pol I transcription machinery remains associated with the rDNAs in the NORs (nucleolar organizer regions). The presence of the pol I subunits, as well as of the SL1 complex, TTF1 and UBF was demonstrated in mitotic NORs corresponding to rDNAs actively transcribed during the previous interphase.[33,34] As detected by electron microscopy, UBF is localized on extended (not condensed) fibers of rDNAs in the NORs,[19] and the DNA-binding capacity of UBF is required for this association.[35] The traffic of transcription factors between rDNA clusters and the cytoplasm is maintained throughout mitosis and varies at different periods of mitosis.[35] Additionally, during metaphase, pol I subunits may transiently dissociate from NORs.[23,35] Thus both the disorganization of the nucleoli and the arrest of rDNA transcription occur in prophase but the release of the nucleolar processing complexes precedes the inactivation of transcription (**Fig. 1**). Due to recent progress in measuring interactions and activities in vivo, the regulation of these events can now be better understood (see below).
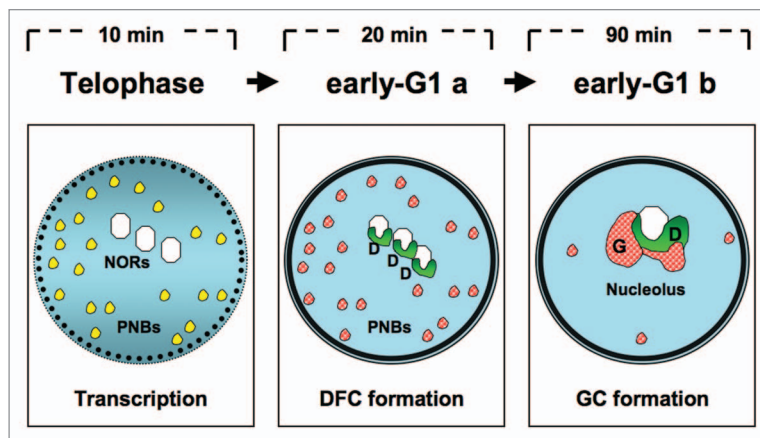
Cell cycle control of nucleolar disassembly. At the beginning and during mitosis, several pol I-specific transcription factors are phosphorylated by the Cdk1-cyclin B kinase; these

phosphorylations are necessary to establish and maintain the suppression of rDNA transcription.[34,36,37] Similarly, phosphorylation of the nucleolar processing proteins is modified during mitosis.[38] The NPM/B23 protein, a component of the pre-ribosomal ribonucleoprotein (RNP) complexes [31,39] is a target of the Cdk1-cyclin B kinase during mitosis.[40] Phosphorylation of NPM/B23 starts in early prophase and is reversed by PP1 phosphatases during anaphase.[40] This phosphorylation decreases the RNA-binding affinity of NPM/B23 [41] and might participate in release of this protein from the nucleolus during prophase.[40] Thus rDNA transcription and rRNA processing complexes appear to be repressed by the same pathway during mitosis. However different timings should be considered in prophase since the most recently synthesized pre-rRNAs accumulate as partially processed 45S pre-rRNAs indicating that total repression of pre-rRNA processing occurs prior to total repression of rDNA transcription.[30] Recently, Gavet and Pines [22] have developed a FRET-based biosensor to measure the activation of the Cdk1-cyclin B kinase pathway in living HeLa cells, and they demonstrated that different levels of Cdk1-cyclin B kinase activity trigger different mitotic events during prophase.[22] In particular, these authors found that nucleolar disassembly requires high levels of Cdk1 activity before breakdown of the nuclear envelope. It is tempting to propose that the different timing for shutdown of nucleolar processing and of rDNA transcription is due to different local concentrations of Cdk1-cyclin B activity.
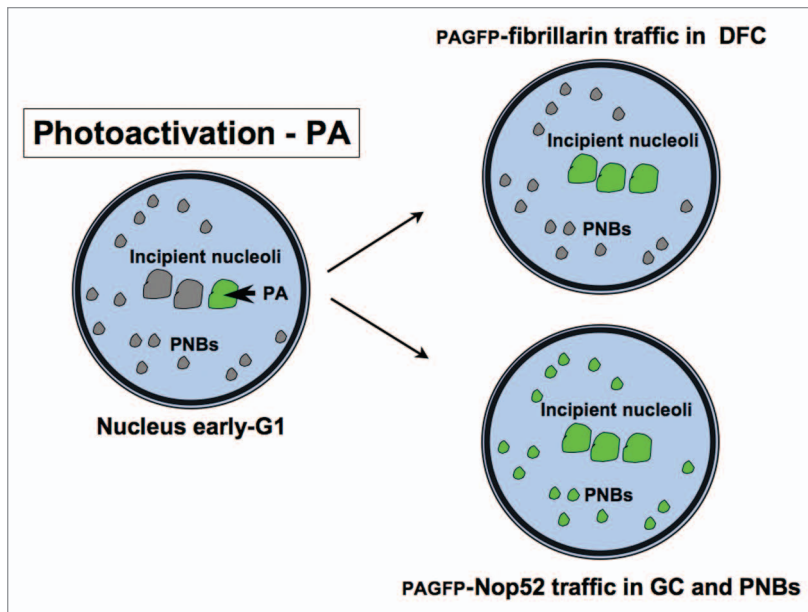

Figure 2. Schematic illustration of nucleolar assembly at the end of mitosis. In telophase transcription of the rDNAs is activated (white octagons) in several NORs (nucleolar organizing regions), whereas the early and late nucleolar processing complexes are located in the prenucleolar bodies (PNBs), consequently the PNBs containing both the D and G processing complexes (respectively D: green and G: red) appeared as yellow structures. The assembly of the nuclear envelope around chromatin is not complete. In early-G$_1$ a, transcription and early processing take place and generate the DFC (D: green), the late processing complexes are in PNBs (red), and the nuclear envelope is assembled at the periphery of the nucleus (double black rings). In early-G$_1$ b, the late processing complexes are recruited around the DFC (D) forming the granular component (G: red) and the incipient nucleoli form a single nucleolus. In early-G$_1$ b, a few PNBs are still present and cytokinesis is not complete (not shown). In Hela cells the duration of the telophase is ≈ 10 min, early-G$_1$ a is ≈20 min and early-G$_1$ b is ≈90 min.

The Dynamics of Nucleolus Assembly Is Time- and Space-Regulated

Nucleolar assembly is an early event starting at telophase, and a complex process that occurs during a relatively long period of the cell cycle. In HeLa cells, of a total of 22 h for one cycle, complete nucleolar assembly takes about 2 h[42] whereas visible nucleolar disassembly requires 30 min.[22] Nucleolar assembly depends on the coordination between activation of rDNA transcription, and recruitment and activation of the RNA processing complexes.[43] In addition, translocation of the rRNA processing complexes into the sites of rDNA transcription is linked to the formation of foci designated prenucleolar bodies (PNBs).[44,45] Therefore, the building of new nucleoli at the beginning of interphase involves the activation of the transcription machinery already in association with rDNA genes, the formation of transient bodies, the PNBs, in the nuclear volume, and the translocation of the RNPs complexes from the PNBs to the transcription sites (**Fig. 2**).

Activation of the transcription. Pol I transcription is first detected during telophase in human cells and late anaphase in PtK1 cells.[27,46] The resumption of rDNA transcription occurs simultaneously in each of the NORs associated with the pol I machinery, 6 competent NORs out of 10 NORs in HeLa cells[33] or in 2 NOR-bearing chromosomes in PtK1 cells.[19] The level of transcription is directly related to the amount of pol I factors

associated with each NOR.[33] At early G$_1$ in HeLa cells, the fusion of two nucleoli into one single nucleolus is observed in living cells in less than 3 min after their contact.[32] This behavior explains the mean number of 2–3 nucleoli in interphase compared to the 6 competent NORs organizing incipient nucleolus in early G$_1$. The mechanism of this fusion is presently unknown. Recently, the fusion of nucleoli was attributed to the liquid-like behavior of nucleoli in *Xenopus laevis* oocytes.[47] To what extent this concept of liquid-like behavior could be applied to other nucleoli (not generated around extra-chromosomal rDNAs as in oocytes) is presently an open question?

During mitosis, pol I transcription is repressed by the CDK1-cyclin B kinase activity, and at the end of mitosis pol I transcription activation depends on inhibition of this activity.[34,36,37] The inhibition of CDK1-cyclin B kinase activity is controlled by the phosphatases PP1 and PP2A.[48] The interruption of inhibition of rDNA transcription is the result of the equilibrium between phosphorylation/dephosphorylation and local distribution of these enzymes occurring during mitotic progression and leading to positive feedback of the Cdk1-inducing mitosis.[49]

Recruitment of the processing complexes via PNB formation. The signature of nucleolar assembly is based on the detectable resumption of rDNA transcription. However, it was demonstrated that active rDNA transcription does not possess the ability to organize a complete nucleolus. Nucleolar assembly also depends on the proteins and snoRNAs of the processing complexes, r-proteins as well as the 45S rRNA produced during prophase and present in the perichromosomal compartment.[30,37,46,50]

Figure 3. Schematic illustration of the traffic of nucleolar processing proteins during nucleolar assembly in incipient nucleoli. The traffic of processing proteins tagged with photoactivatable green fluorescent proteins (PAGFP) activated by laser (PA) was analyzed using fibrillarin for DFC marker and using PAGFP-Nop52 for GC marker. The pool of PAGFP-fibrillarin activated in one incipient nucleolus (green) migrates to the other incipient nucleoli but not to PNBs. The pool of PAGFP-Nop52 activated in one incipient nucleolus (green) migrates to the other incipient nucleoli and also to PNBs. This explains the progressive formation of GC controlled by PNBs as described in reference 42.

During nucleolar assembly, these nucleolar processing complexes assemble in foci designated PNBs (**Fig. 2**). In 3-D time-lapse imaging starting in metaphase, the first detectable concentration of processing proteins in PNBs was observed during telophase on the chromosome surface.[32] The PNBs are present during early G_1 a and G_1 b, periods lasting about 2 h in HeLa cells.[42] Their fibro-granular heterogeneous structure and their association with condensed chromatin were characterized by electron microscopy.[27,32] In the PNBs formed during telophase, early and late nucleolar processing proteins, snoRNAs, r-proteins and unprocessed 45S rRNAs are colocalized and PP1γ phosphatase is present.[26,30,42,45,50-52] In some cells containing abundant nucleolar processing complexes, the formation of foci designated nucleolus-derived foci (NDFs) are also observed in the cytoplasm during anaphase and telophase.[53,54]

The colocalization of proteins in the same structure does not indicate whether or not these proteins interact. Therefore, to determine when and where the interactions between processing proteins of the same complexes occur, FRET was set up to analyze the dynamics of these interactions in living cells.[55] We demonstrated that the interactions between NPM/B23 and Nop52 are detectable in nucleoli and also in PNBs.[26] Interestingly, no interaction is detected during anaphase at the periphery of the chromosomes, whereas they are registered in about 20% of the PNBs at the beginning of telophase, 40% at the end of telophase and 55% in early G_1 nuclei.[26] It has as yet not been established whether these interactions lead to processing of the ITS2 (internal transcribed spacer 2) of pre-rRNAs present in the

PNBs. However the presence of PP1γ phosphatase in PNBs is compatible with reversible mitotic phosphorylation inducing pre-rRNA cleavages.[56]

The flux of processing proteins between the rDNA transcription sites and PNBs was measured in living cells at different periods of nucleolar assembly using photoactivation (PA) (**Fig. 3**). The principle is to render nucleolar PAGFP-tagged proteins fluorescent by photoactivation to mark a pool of proteins and then follow the kinetics and distribution of these molecules.[57] PAGFP-fibrillarin, PAGFP-NPM/B23 or PAGFP-Nop52 can be photoactivated and become visible in one PNB but in each case the amount was too low to analyze their 3-D distribution. Interestingly, the pool of PAGFP-fibrillarin activated in one incipient nucleolus migrated to the other active NORs and was not detectable in PNBs.[42] This illustrates the association of fibrillarin with the early processing steps of pre-rRNAs present in the 6 incipient nucleoli and suggests that these pre-rRNAs are absent or in very limited quantity in PNBs. The progressive exclusion of fibrillarin from PNBs in 20–30 min indicates that the early processing cleavage might occur in PNBs. On the contrary the proteins of late rRNA processing (Nop52 or NPM/B23) migrate from one incipient nucleolus to the others and also to each PNB (**Fig. 3**). The feedback of the proteins between nucleoli and PNBs is achieved 2 h after telophase in normal conditions and PNBs are no longer visible. This is the consequence of the increased number of binding sites in incipient nucleoli when the first step of nucleolar assembly is accomplished. In contrast, the persistence of PNBs and traffic between PNBs and incipient nucleoli is the signature of disturbed nucleolar assembly.[42] Thus, the recruitment of the processing complexes, first in the DFC (fibrillarin) and then in the GC (Nop52) during nucleolar assembly after mitosis depends on the traffic between PNBs and incipient nucleoli (**Fig. 3**). Consequently PNB formation is a way of controlling and regulating nucleolar assembly after mitosis and might explain the ubiquitous formation of PNBs in open mitosis.

Outlook and Perspectives

The nucleolus is a model of coordination between several networks that must cooperate to produce the 40S and 60S ribosome subunits. These networks are in charge of the activation of pol I transcription in competent NORs to synthesize the 47S rRNAs, of the recruitment of the processing complexes on the 47S rRNAs and of the assembly of the processed rRNAs with about 80 r-proteins. Presently most of the studies analyzing nucleolar assembly/disassembly have been focused on the activation/repression of transcription and of the rRNA processing complexes while the role of the r-proteins has not been explored. However, it is noticeable that the mutation of one of the r-proteins in Diamond-Blackfan anemia affects the function of the proteins in rRNA

processing.[58] How the r-proteins participate in nucleolar assembly after mitosis is an open question.

Another way that would benefit from a more thorough investigation is the coordination between assembly of the nucleoli and other nuclear domains, in particular the Cajal Body in which snoRNP complexes are assembled. There is no doubt that ribosome biogenesis depends of snoRNP complexes in particular the U3 snoRNP responsible of the folding of pre-rRNAs necessary for the assembly of the small ribosomal subunit.[59] Likewise, the traffic between CB and incipient nucleoli is necessary to coordinate the early and late steps of ribosome biogenesis that makes GC formation possible.[42] To what extent the kinetics of nucleolar assembly depends on the function of the Cajal body is presently an open question.

After mitosis, the progressive recruitment of the processing proteins into nucleoli is regulated by the dynamics of the exchanges between PNBs and incipient nucleoli.[42] This observation supports the hypothesis that the nucleolar processing complexes localize in these 2 sites by binding to pre-rRNAs. What is the destiny of the pre-rRNAs present in PNBs is presently unclear. These pre-rRNAs could be processed in PNBs, exported to the cytoplasm as ribosome subunits, recruited into new nucleoli or degraded. During Xenopus embryogenesis, PNBs were formed after mitosis in the absence of pol I transcription.[60] In these nuclei, the pre-rRNAs and nucleolar proteins from PNBs localized around the rDNAs before the detection of the pol I complex.[52] Thus, the regroupment of PNBs around the rDNAs can occur in the absence of pol I transcription during Xenopus embryogenesis. In HeLa cells in which pol I transcription is drug-inhibited during the transition mitosis/interphase, the processing proteins and the pre-rRNAs are observed around the rDNAs.[30] Hence, most probably the PNBs are not only involved in the timing of recruitment of nucleolar complexes during nucleolar assembly and could explain that these transitory bodies are ubiquitous in open mitosis. Why this step has been conserved throughout evolution is still to be established.

Until now, the rational has been to investigate the assembly of the nucleolus only considering the point of view of ribosome biogenesis even though the nucleolus is a plurifunctional domain;[11] most probably other players that participate in nucleolar assembly have been neglected. According to nucleolar proteome analyses, the ribosome machineries would correspond to ≈40% of the total nucleolar proteins,[61] and genomics predict that 4% of the genes are associated with the nucleoli.[62,63] At the time of the initiation of transcription during the building of DFC around FC, EM observations demonstrated that the incipient nucleoli are in contact with the nuclear envelope in human cells[64] and the contact of the nucleolus with the nuclear envelope is maintained all along interphase in many cell types.[65] Recently it was demonstrated that lamin B1 maintains the functional plasticity of nucleoli [66] and participates in the post-mitotic structural reorganization of the nucleus and nucleoli.[67] In correlation with these conclusions, DNA sequencing of the nucleolus-associated chromatin domains revealed chromatin loci specifically associated with either the nucleolus or the nuclear envelope.[63] Interestingly, the reorganization of the nuclear envelope after mitosis is achieved with the same timing as nucleolar assembly.[68] In some models of nuclear organization, the nucleolus is proposed to contribute to chromosome gathering.[69] To what extent nucleolar assembly orders the nuclear compartment in nuclei remains to be investigated but this could be one possibility as the fusion of incipient nucleoli are observed in early G_1, gathering several chromosomes in the same domain.

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